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Short communication

Larval immersion tests with ivermectin in populations of the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from State of Sao Paulo, Brazil

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Abstract

Larval immersion tests (LIT) with commercial formulation of ivermectin were carried out with larvae of two field populations of *Rhipicephalus (Boophilus) microplus* from commercial dairy farms of the State of Sao Paulo, Brazil and a susceptible strain (Porto Alegre) to differentiate resistant-suspected and susceptible strains. One of the populations tested (Barra Alegre) showed a LC_{50} value significantly higher than the susceptible strain and a resistance ratio (CI95%) of 3.78 (3.47–4.12), leading to suspect that this population shows traces of resistance to ivermectin. Population Sao Francisco, with no records of ivermectin injections on cattle, showed no difference on the ivermectin response in relation to Porto Alegre strain characterizing it as susceptible. Although LIT is not yet recommended by FAO to diagnose resistance to acaricides, this technique was successful on the differentiation of resistance-suspected population and a susceptible strain and can be used to detect populations of *R. (B.) microplus* resistant to ivermectin. This is the first report of *R. (B.) microplus* resistant to ivermectin detected by an in vitro bioassay.

Keywords: *Rhipicephalus (Boophilus) microplus*; Cattle tick; Resistance; Ivermectin; Bioassays

1. Introduction

Rhipicephalus (Boophilus) microplus is among the most important cattle ectoparasites in the tropical and subtropical areas of the world. In Brazil, this ixodid tick infests approximately 80% of the bovine population and this degree of infestation is responsible for high economic losses, which represents about two billion dollars a year in Brazil (Grisi et al., 2002).

The control of this parasite is performed mainly through chemical acaricides. However, continuous use of acaricides has led to the problem of resistance in these arthropods. Worldwide, cases of resistance to the different acaricide classes include organophosphates (Ortiz-Estrada et al., 1995; Baxter et al., 1999; Davey et al.,

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2006), synthetic pyrethroids (Schnitzerling et al., 1989; Arantes et al., 1996; Davey and George, 1998; Davey et al., 2006) and formamidines (Benavides Ortiz, 1995; Vargas et al., 2003; Davey et al., 2006). Recently, one case of a *R. (B.) microplus* strain resistant to macro-cyclic lactones (MLs) was reported by Martins and Furlong (2001). The occurrence of resistance to MLs have been reported in nematodes (Njue and Prichard, 2004) and other arthropods (Scott, 1989; Clark et al., 1995).

MLs are represented by two groups, avermectins (ivermectin, abamectin and doramectin) and milbemycins (moxidectin and milbemycine oxime), largely used in Brazil for the control of internal (gastrointestinal nematodes and microfilariae) and external (ticks, mites and mange) parasites. MLs are now 12% of the total Brazilian animal health market (SINDAN, 2005). The mode of action of these compounds in arthropods is attributed to their high affinity to glutamate-gated chloride channels (*Glu-Cl*) that are present in muscle and nerves. The opening of these channels causes a slow and irreversible membrane conductance increase, resulting in somatic musculature paralysis and the consequent death of the parasite (Cully et al., 1994).

Early detection of resistance is essential in order to avoid further selection of resistant ticks using the same active ingredient and to delay the spread of resistance. To detect *R. (B.) microplus* populations resistant to acaricides, Food and Agriculture Organisation (FAO) has recommended bioassays, adult immersion test (AIT) and larval package test (LPT), which can be done at low costs. Essentially, differences among these tests are the utilization of individuals at different instars and their exposure time to treatment. For more than 40 years, bioassays have been used with success for the detection of resistance (FAO, 2004).

The larval immersion test (LIT), or Shaw larval immersion test (Shaw, 1966), was modified by Sabatini et al. (2001) and used to test Australian strains of *R. (B.) microplus* with macrocyclic lactones to determine 50 and 99.9% lethal concentrations and set a discriminating dose (DD) that could be used to diagnose LMs resistance. In that paper, the authors noted the high sensitivity of the test but also that the determined DD may only be useful to test Australian populations and that more tests should be performed with other tick populations. Benavides and Romero (2000) in Colombia used LIT method to evaluate the ivermectin action on an acaricide multiresistant reference strain (Montecitos) and a susceptible reference strain (Yeerongpilly). Nevertheless, they found no significant differences between the responses of the two strains. In both publications, the authors mention that the LIT is not yet recommended by FAO as a diagnostic method for resistance and that further assays should be conducted with this technique to assess its performance for differentiation of susceptible and resistance-suspected populations.

Because of the need for more information on the use of LIT as a method to diagnose resistance to MLs in *R. (B.) microplus* populations, allied with the existence of only one report of MLs resistance worldwide and the recent producers complaints about ivermectin efficacy on cattle tick control in Brazil, LITs were performed with a Brazilian susceptible reference strain (Porto Alegre) and a field strains of *R. (B.) microplus* derived from commercial dairy farms in the State of Sao Paulo, where resistance to ivermectin was suspected.

2. Materials and methods

2.1. Ticks

R. (B.) microplus populations were collected in two dairy farms at the municipalities of Ibiúna (S23°41′15″–W47°11′15″) – population Sao Francisco, and Piquete (S22°36′59.0″–W45°08′23.4″) – population Barra Alegre, located in the State of Sao Paulo, Brazil. Barra Alegre was chosen due to the use of ivermectin for cattle tick control in the last 5 years and recent difficulties with control. As a comparison, population Sao Francisco was selected and it had no history of ML applications.

R. (B.) microplus susceptible reference strain Porto Alegre (POA) (provided by Dr. Itabajara da Silva Vaz Jr.; Federal University of Rio Grande do Sul, Brazil) was used as a control.

2.2. Preparation of ticks

About 100 engorged females were collected directly from infested animals, placed in identified carton boxes and transported to the laboratory at the University of Sao Paulo. They were washed in distilled water and allowed to dry in paper towels, then weighted and stuck to the lid of a plastic Petri dish (4.5 cm diameter, 1.5 cm high) with double-sided sticky tape. Ticks were kept at 27–28 °C and 80–90% relative humidity. After 14 days, eggs were collected and transferred to a plastic conic tube (50 mL) with a cotton lid to allow air and moisture exchange. After another 2 weeks the eggs hatched and after a further 2 weeks the larvae were ready for testing.

2.3. Larval immersion test (LIT)

A commercial formulation of ivermectin 1% (IVOMEC©–Merial Saúde Animal, Brazil. Batch number

195/03, expiration date 08/2007) was diluted in 1% of an ethanol 100%–TritonX–100 2% solution (Eth–TX 2%) in distilled water in order to prepare immersion solutions. The concentrations of ivermectin in immersion solutions were 0.0000625; 0.000125; 0.00025; 0.0005; 0.001; 0.0025; 0.005; 0.01% and the ethanol concentration was 1% and TritonX-100 was 0.02%. Solutions, 1 mL each, were prepared in 1.5 mL microcentrifuge tubes and then approximately 500 larvae were added to each one. Control solutions were prepared adding 10 μL of Eth–TX2% in 990 μL of distilled water.

Immediately after addition of larvae, the tube was closed and shaken vigorously for some seconds and then gently for 10 min. The tubes were then opened and the larvae transferred with a paintbrush to a paper filter. After drying, about 100 larvae were transferred to a squared paper filter (850 mm × 750 mm) that was folded and closed with ''bulldog'' clips forming a packet. The packets were incubated at 27–28 °C and 80–90% relative humidity for 24 h then the mortality was determined. Only larvae capable of locomotion were considered alive. Bioassays were in triplicate.

The mortality data were submitted to a probit analysis and a chi-square test was used to test the hypotheses of parallelism and equality ($P = 0.05$) with POLO PLUS software (LeOra Software, 2003) to calculate the lethal concentrations (LC) for 50 and 99% with their respective confidence intervals of 95% (CI95%). Resistance ratios (RR) were calculated in relation to POA strain (susceptible) and significance of each comparison was determined only if one was not included in the confidence interval. DD was set as $2 \times LC_{99}$ calculated from the POA strain analysis.

3. Results

The LC_{50}/LC_{99} values for ivermectin with their respective CI95% and slopes for each population are shown in Table 1. It was observed that the population Barra Alegre, that had been exposed to MLs at least for

Table 1
Lethal concentrations for 50 and 99% and resistance ratios of 14–21 days old larvae of *R. (B.) microplus* populations from Ibiuna and Piquete municipalities (State of São Paulo, Brazil) and the Porto Alegre strain, calculated from the larval immersion tests with a commercial formulation of ivermectin 1%

Population	Slope ± S.E.*	LC_{50} (95% CL) (%), LC_{99} (95% CL) (%)	RR** (LC_{50}) (95% CL), RR (LC_{99})(95% CL)
Porto Alegre strain#	6.317 ± 0.318	0.00042 (0.00039–0.00045), 0.00098 (0.00085–0.00120)	–
São Francisco	5.536 ± 0.233	0.00046 (0.00043–0.00049), 0.00122 (0.00108–0.00142)	1.09 (1.03–1.16), 0.00098 (0.00085–0.00120)
Barra Alegre	2.065 ± 0.065	0.00160 (0.00135–0.00192), 0.021 (0.014–0.036)	3.78 (3.47–4.12), 21.71 (17.42–27.05)

All tests were conducted in triplicate.
Susceptible control.
* Standard deviation.
** Resistance ratio.

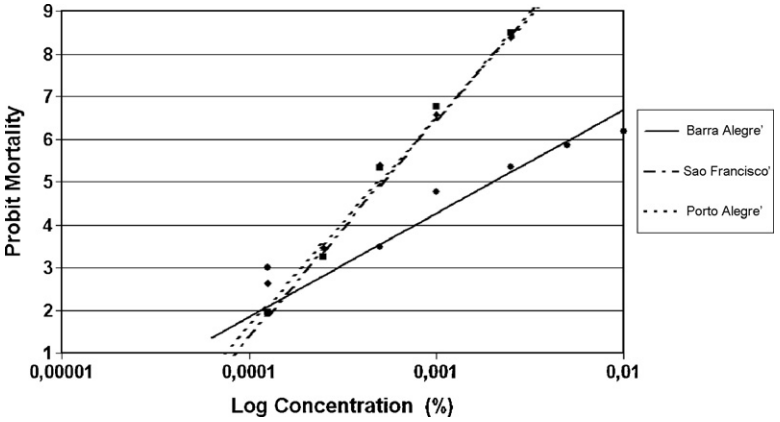

Fig. 1. Probit mortality × log concentration plots from *R. (B.) microplus* Porto Alegre strain, Sao Francisco population and Barra Alegre population, submitted to larval immersion test with commercial formulation of ivermectin diluted in ETH-TX.

5 years, had significantly higher LC_{50}/LC_{99} values than the control (Porto Alegre strain).

Fig. 1 presents ivermectin probit mortality × log concentration plots and regression lines of the Porto Alegre strain (susceptible), Barra Alegre population and the Sao Francisco population. The LC_{50}(CI95%) calculated for Sao Francisco population, 0.00046% (0.00043–0.00049%), was not different when compared to the LC_{50}(CI95%) value calculated for the Porto Alegre strain, 0.00042% (0.00039–0.00045%). Barra Alegre showed a higher LC50 value than Porto Alegre strain and Sao Francisco population (0.00160% [0.00135–0.00192%]). The hypothesis of parallelism between regression lines for Barra Alegre and Porto Alegre strains was rejected ($P < 0.05$). A smaller slope value from Barra Alegre in relation to Porto Alegre indicates a heterogeneous response to increasing concentrations of ivermectin on population Barra Alegre.

Considering the LC_{99} value calculated for the Porto Alegre strain (0.00098%), the DD for ivermectin was set at 0.002%.

4. Discussion

Development of resistance to drugs is an evolutionary adaptation that puts at risk every parasiticidal agent (Shoop, 1993). Resistance can be defined as a significant increase in the number of individuals within a single parasite population that can tolerate doses of a drug(s) that have proven to be lethal for most individuals of the same species (FAO, 2004). An important factor in resistance emergence in a population is the frequent use of the same product for a long period of time, which selects resistant individuals by enhancing the frequency of a resistance gene(s), leading to lack of efficacy of this product (Sutherst and Comins, 1979)

Macrocyclic lactones (avermectins and milbemycins) are the most used drugs to control both internal and external parasites in Brazil. In 2004, about 350 million doses were used, representing 12% of sales of animal health products (SINDAN, 2005). These numbers illustrate the economic importance of macrocyclic lactones in Brazil and help to justify the research efforts on resistance diagnosis and control. Considering that ivermectin, the most representative drug of MLs class, has been used in Brazil since the 1980s, some populations of *R. (B.) microplus* could have developed resistance to this molecule where it has been used extensively. In fact, Martins and Furlong (2001), using field trials, reported for the first time in Brazil a *R. (B.) microplus* ML (doramectin) resistant strain (Sao Gabriel strain).

LIT was conducted with Porto Alegre strain (susceptible) for the determination of LC_{50}/LC_{99} and DD. The calculated LC_{50} (CL95%) value – 0.00042% (0.00039–0.00045%) – is close to LC_{50} value determined for the Yeerongpilly strain (from Australia) by Sabatini et al. (2001)—0.00041% (0.000097–0.000729%). We can conclude that the toxicity of ivermectin in Australian and Brazilian susceptible strains is similar. Comparing the discriminating dose proposed by Sabatini et al. (2001) with our results again shows little difference, 0.0027% for the Australian and 0.0020% for the Brazilian ones.

Slope values are another indicative data for resistance. Smaller slopes are common in field collected resistant strains and population Barra Alegre presented a lower slope value than Porto Alegre. The susceptible population showed a more uniform response to increasing concentrations of ivermectin than the resistant-suspected population. Maybe this is happening due to the presence of both resistant and susceptible genes in the Barra Alegre population leading to the presence of both heterozygous and homozygous individuals in this resistant population.

To validate the proposed bioassay in the field, the LIT was performed with a population suspected of resistance to ivermectin and the LC50/LC99 values calculated. The data analysis showed significant differences between the LC50 values of the studied population and Porto Alegre strain. There is a difference in the ivermectin toxicity between the field population (Barra Alegre) and the susceptible strain. Fig. 1 shows that a proportion of the Barra Alegre population (approximately 50%) are resistant. This may be related with resistance at this property, as there was a suspicion of resistance to ivermectin because of poorer tick control. We can assume that the LIT is capable of differentiating populations in relation to response to ivermectin treatment. In the case of Sao Francisco population, from Ibiuna/SP, which has never been exposed to LMs, the LC_{50}/LC_{99} values showed no significant difference to Porto Alegre strain (Fig. 1). These facts help us to conclude that LIT is efficient for differentiating resistant and susceptible strains. As pointed out by Sabatini et al. (2001), the value of this diagnostic test could only be verified if or when resistance to MLs emerges in ticks. Although, not yet recommended by the FAO, our data show that LIT is a sensitive test and we can assume that it can be used for diagnosis of resistant populations of *R. (B.) microplus* to ivermectin.

The survey conducted at the Vale do Paraiba region in this study indicates that there are tick populations developing resistance to ivermectin and this may be occurring in other parts of Brazil. It is therefore,

important that further studies be conducted, particularly to relate loss of tick control using MLs with the results of the LIT. For this, it will also be necessary to develop a laboratory strain with homozygous ivermectin resistance.

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